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Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease

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To explore the interactions between regulatory T cells and pathogenic effector cytokines, we have developed a model of a T cell-mediated systemic autoimmune disorder resembling graft-versus-host disease. The cytokine responsible for tissue inflammation in this disorder is interleukin (IL)-17, whereas interferon (IFN)- γ produced by Th1 cells has a protective effect in this setting. Because of the interest in potential therapeutic approaches utilizing transfer of regulatory T cells and inhibition of the IL-2 pathway, we have explored the roles of these in the systemic disease. We demonstrate that the production of IL-17 and tissue infiltration by IL-17-producing cells occur and are even enhanced in the absence of IL-2. Regulatory T cells favor IL-17 production but prevent the disease when administered early in the course by suppressing expansion of T cells. Thus, the pathogenic or protective effects of cytokines and the therapeutic capacity of regulatory T cells are crucially dependent on the timing and the nature of the disease.

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Immune-mediated inflammatory diseases often present with a combination of destructive tissue inflammation and various systemic manifestations. It has been generally believed that most such disorders are caused by unregulated activation of T helper (Th)1 cells, which produce the proinflammatory cytokines IFN- γ and TNF. The success of TNF antagonists for treating several of these inflammatory diseases is a striking example of rational immune therapy based on the identification of a key pathogenic cytokine (1, 2). Recent studies indicate that the cytokine IL-17 is the major mediator of tissue inflammation in several models of inflammatory disease (3–9). These findings have led to studies aimed at defining the control of IL-17 production and its pathologic actions. There is also great interest in immunosuppressive approaches targeting the IL-2 pathway and in the possible use of regulatory T (T reg) lymphocytes for treating immune-mediated inflammatory diseases (10–12). To realize this potential, it will be necessary to define the types of pathological immune reactions that can be controlled or reversed by T reg lymphocytes. In fact, recent studies have shown that T reg lymphocytes do not suppress, and

may even enhance, IL-17 production by T cells, an effect that is likely mediated by TGF- β , which is produced by T reg lymphocytes and is a stimulus for IL-17 production (13–15). Such results have raised the possibility that T reg lymphocytes may not be useful for immune-mediated inflammatory diseases in which IL-17 plays a central role.

RESULTS AND DISCUSSION

To address the roles of cytokines and T reg lymphocytes in immune-mediated inflammation, we have established a model in which a systemic autoimmune disease is caused by a monospecific T cell population that can be followed quantitatively in vivo. In this model, CD4 T cells from the DO11.10 (DO11) T cell receptor transgenic mouse, specific for the ovalbumin (OVA)_{323–339} peptide, are transferred into a lymphopenic (Rag^{-/-}) host expressing OVA as a secreted, systemic antigen. The transferred T cells expand, develop into Th1 effector cells, and cause a severe disease characterized by weight loss and skin inflammation (16). The disease has many similarities to graft-versus-host disease (GvHD), in which transferred T cells react against host antigens in an environment deficient in endogenous lymphocytes. Since the signature cytokine of Th1 cells is IFN- γ , we first asked if IFN- γ was responsible

J. Lohr and B. Knoechel contributed equally to this work.
The online version of this article contains supplemental material.

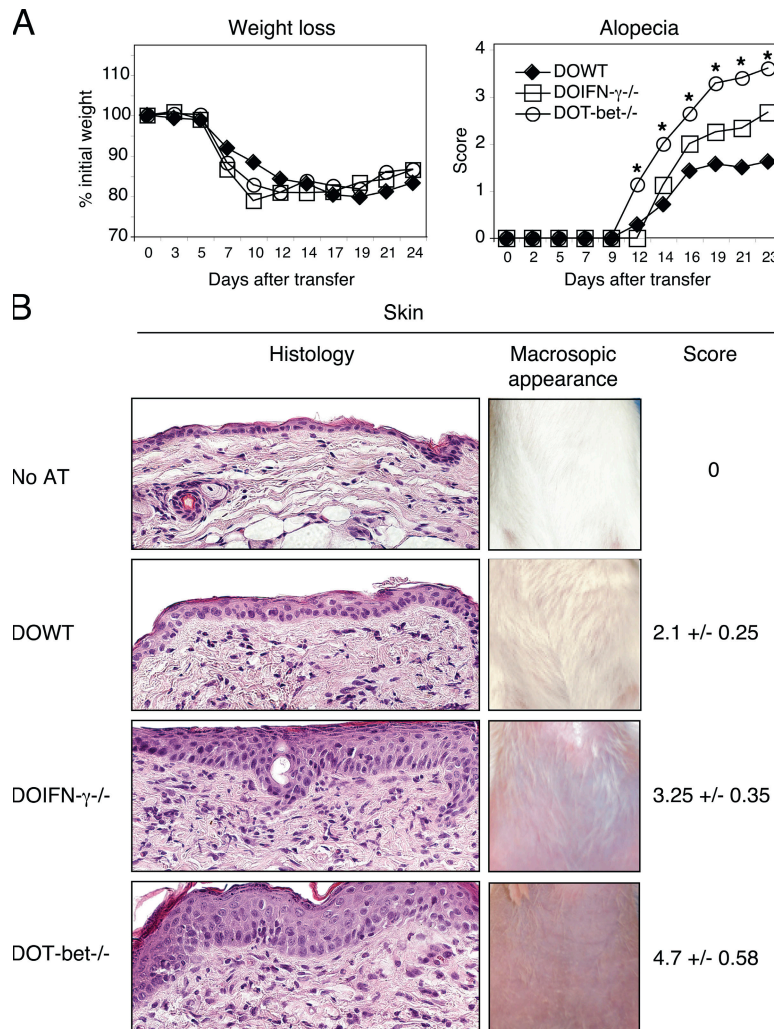


Figure 1. Role of IFN- γ and T-bet in the systemic immune reaction. CD4⁺KJ1-26⁺CD25⁻ cells were isolated from the lymph nodes and spleens of WT, IFN- γ ^{-/-}, or T-bet^{-/-} DO11 mice by cell sorting, and 10⁶ cells were transferred into sOVA Tg Rag^{-/-} mice on day 0. (A) Body weight is plotted over time as the percentage of the starting weight before T cell transfer. Data are pooled from six to eight mice per group. Alopecia was scored as described in Materials and methods and plotted

over time. Data are pooled from six to eight mice per group (*, $P < 0.05$ compared with WT DO11 cells). (B) Skin of lower abdomen is shown on day 23 after transfer for one representative mouse in each group. H&E stain of skin sections is shown on the left, and macroscopic appearance of the skin is shown in the middle. Histological scores were obtained as described in Materials and methods and averaged from three to four mice per group \pm SD.

for the disease. To do this, we compared the pathogenic effects of wild-type (WT) DO11 cells with those of DO11 cells lacking either IFN- γ or the Th1-specific transcription factor, T-bet (17). Ablating the Th1 response did not ameliorate weight loss and, surprisingly, led to more severe inflammatory skin lesions (Fig. 1). The relative severity apparent from the external appearance and histology was corroborated by scoring the lesions (Fig. 1 B) as described in Materials and methods. Cell numbers in lymphoid organs of recipient mice that received WT DO11 cells were similar to recipients that were transferred with either IFN- γ ^{-/-} or T-bet^{-/-} DO11 cells (unpublished data). IFN- γ has been suggested to play a role in the development and/or function of T reg lymphocytes (18). To ask if the exacerbated disease in the absence of

IFN- γ could reflect a failure of endogenous T reg lymphocytes development, we followed the DO11 cells for generation of CD25⁺FoxP3⁺ T reg lymphocytes. These assays showed no defect in the numbers of T reg lymphocytes in the absence of IFN- γ or T-bet (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20061341/DC1>).

We have begun to identify which cell populations infiltrate the skin in sOVA transgenic (Tg) Rag^{-/-} recipients by isolating cells from skin and flow cytometry. Fig. S2(available at <http://www.jem.org/cgi/content/full/jem.20061341/DC1>) shows that the skin is infiltrated by CD3⁺KJ1-26⁺ T cells and Gr-1⁺CD11b⁺ granulocytes. This is not the case in normal naive DO11 Rag^{-/-} mice or normal sOVA Tg Rag^{-/-} mice that have not received DO11 cells.

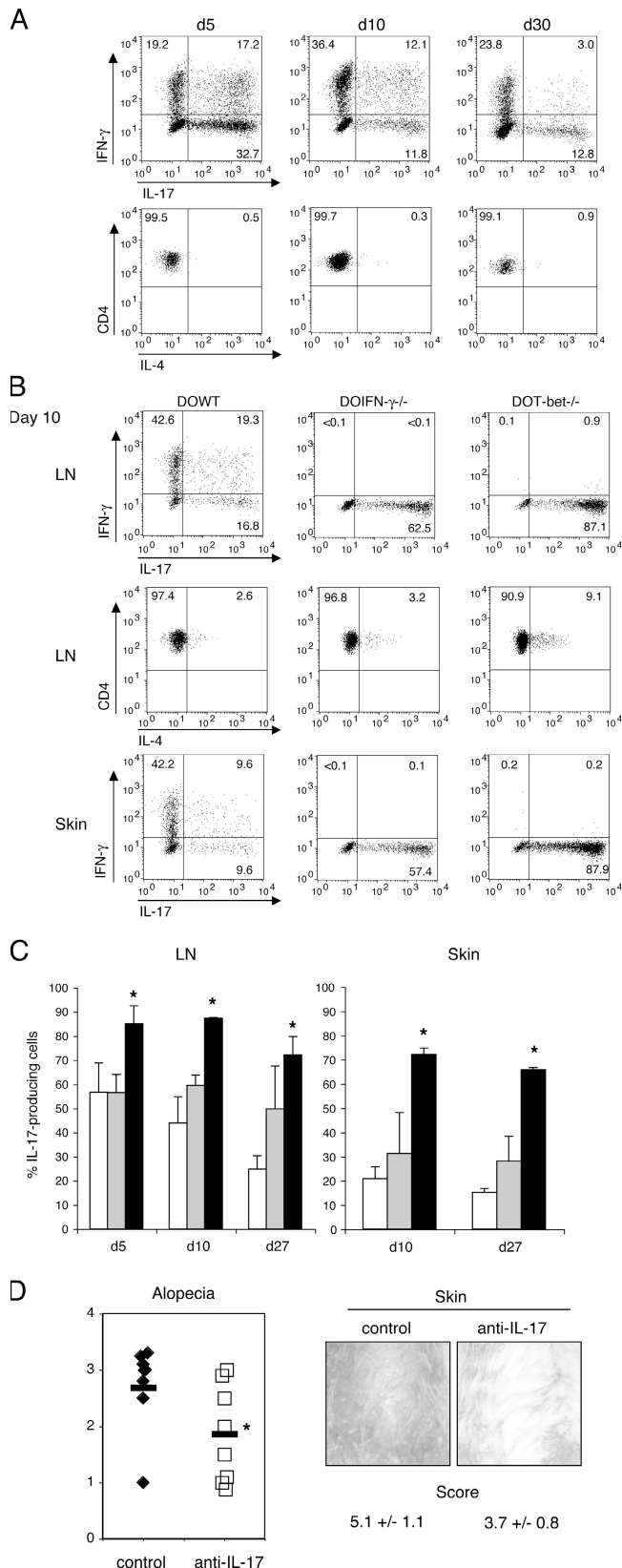


Figure 2. Kinetics and regulation of IL-17 production in systemic autoimmunity. (A) 10^6 purified CD4 $^{+}$ DO11 Rag $^{-/-}$ cells were transferred

It is possible that the inflammatory disease is caused by the cytokine IL-17, and production of IL-17 is increased in the absence of IFN- γ (19, 20). Because the entire reaction in this model involves one T cell population, one can easily follow the profiles of cytokines produced by these cells. Intracellular cytokine stains showed that early during the course of the disease (5 d after initiation of the reaction), there were populations of activated T cells in lymphoid organs that produced either IFN- γ or IL-17 or both cytokines. Over time, the IL-17 producers decreased in number (Fig. 2 A). Strikingly, in the absence of IFN- γ or T-bet, the number of IL-17-producing T cells did not decline in lymphoid organs or in the skin (Fig. 2, B and C). Only very few IL-4-producing DO11 cells could be found before (day 5–10) or after (day 30) development of skin disease (Fig. 2 A). The correlation between increased skin inflammation and IL-17 production in the absence of a Th1 response suggested that the inflammation was caused primarily by IL-17-producing T cells. In fact, an anti-IL-17 antibody was able to reduce the skin inflammation when DO11 T-bet $^{-/-}$ T cells were transferred into sOVA Tg Rag $^{-/-}$ recipients, as indicated by the severity of alopecia macroscopically and microscopically (Fig. 2 D). These results support a reciprocal relationship between Th1 responses and IL-17 production and indicate that IL-17 is an important cytokine in tissue (i.e., skin) inflammation.

Because of the potential beneficial effect of blocking IL-2 signaling in immunological reactions, we have been interested in the types of pathologic immune responses that are influenced by IL-2. We have previously shown that in the absence of IL-2, i.e., when IL-2 $^{-/-}$ DO11 cells are transferred into sOVA Tg Rag $^{-/-}$ IL-2 $^{-/-}$ recipients, the mice develop a severe, progressive skin disease with massive accumulation of DO11 cells in lymphoid organs and skin. These

into sOVA Tg Rag $^{-/-}$ mice on day 0. Peripheral lymph nodes were harvested at the indicated time points after transfer and stained for intracellular cytokines. All plots are gated on KJ1-26 $^{+}$ CD4 $^{+}$ cells. Numbers refer to the percentage of cytokine-positive cells. Representative plots are shown from one experiment out of four with two to three mice per group. (B) CD4 $^{+}$ KJ1-26 $^{+}$ CD25 $^{-}$ cells were isolated from lymph nodes and spleens from WT, IFN- $\gamma^{-/-}$, or T-bet $^{-/-}$ DO11 mice by cell sorting, and 10^6 were transferred into sOVA Tg Rag $^{-/-}$ mice on day 0. Peripheral lymph nodes and skin were harvested on day 10 after transfer, and the percentage of KJ1-26 $^{+}$ CD4 $^{+}$ cells that express intracellular IFN- γ , IL-17, and IL-4 was measured by flow cytometric analysis. One representative mouse is shown for each group. (C) The percentage of cells producing intracellular IL-17 was determined as in B at the indicated time points. Each bar represents three to four mice from two representative experiments (*, $P < 0.05$). (D) 10^6 purified CD4 $^{+}$ CD25 $^{-}$ DO11 T-bet $^{-/-}$ cells were transferred into sOVA Tg Rag $^{-/-}$ mice on day 0. The mice were injected with anti-IL-17 or control Ig every 3 d. Alopecia was scored on day 16 as described in Materials and methods. Data are pooled from three experiments (*, $P < 0.05$). Macroscopic appearance of the skin of the lower abdomen is shown on day 18 from one representative mouse in each group. Histological scoring was done as described in Materials and methods, and depicted numbers represent averages of six to seven mice per group \pm SD.

mice fail to recover from the disease and this is associated with lack of generation of regulatory T cells (16). To address the role of IL-17 in this reaction, we assayed T cells for IL-17 production in vivo in the absence of IL-2. Surprisingly, large numbers of IL-17-producing T cells accumulated in the skin in the IL-2-deficient setting (Fig. 3). In fact, skin infiltration was dominated by IL-17-producing cells, demonstrating that efficient generation of tissue-infiltrating IL-17-producing cells occurs in the absence of IL-2. Because classical Th1 responses are dependent on IL-2 (21), our findings raise the possibility that IL-17 is an important mediator of the inflammatory disease that develops in knockout mice lacking IL-2 production or signaling (22).

It has been shown recently that T reg lymphocytes promote IL-17 production in vitro, in the presence of inflammatory cytokines such as IL-6 (13). If this happens in vivo, then T reg lymphocytes may not be able to control reactions that are dominated by IL-17. To address this directly, we asked if OVA-specific T reg lymphocytes could prevent the systemic inflammatory syndrome, and how the production of pathogenic IL-17 is affected by the T reg lymphocytes. T reg lymphocytes specific for a defined antigen can be generated experimentally by exposing developing CD4 T cells specific for the antigen to the cognate protein expressed in the thymus. We have shown previously that in crosses of DO11 mice with RIP-mOVA-transgenics, CD25⁺FoxP3⁺ T reg lymphocytes develop, because the antigen is expressed in the thymus and T cells encounter the antigen during maturation (23). CD25^{high}CD4⁺ DO11 T cells were purified from DO11×RIP-mOVA Tg mice and cotransferred with WT DO11 cells expressing a different allelic marker (Thy1.1) into lymphopenic sOVA Tg recipients. The T reg lymphocytes completely prevented the weight loss (Fig. 4 A) and skin inflammation

(Fig. 4 B) that are characteristic of the inflammatory disease. Because we were able to distinguish the T reg lymphocytes from the effector cells, we could determine the effects of the OVA-specific transferred T reg lymphocytes on the response patterns of the effector T cells of the same specificity. The most striking effect of T reg lymphocytes was a profound inhibition of T cell accumulation, which was detectable as early as 5 d after transfer (Fig. 4 C). T reg lymphocytes almost completely inhibited the production of IFN- γ but, surprisingly, enhanced the frequency of IL-17-producing cells among the remaining DO11 cells (Fig. 4 C). However, even though the percentage of IL-17-producing cells was increased within the surviving effector cell population, the total number of cytokine-secreting cells was markedly decreased. Importantly, when T reg lymphocytes were cotransferred with effector cells at a lower ratio, there was no longer an increase in the percentage of IL-17-producing cells (unpublished data) and the total number of IL-17-producing cells was reduced (Fig. 4 D). These data indicate that strong immune responses to a systemic antigen can be prevented when T reg lymphocytes are present at the time of the initial activation, and this protective effect of T reg lymphocytes is dominant over their IL-17-promoting ability. It remains to be determined how T reg lymphocytes inhibit the accumulation of effector cells. A plausible explanation could be competition for IL-2 or other growth factors, or, alternatively, T reg lymphocytes may inhibit IL-2 production and lead to apoptosis of early activated cells.

Whether or not T reg lymphocytes can also have a protective effect if they are generated or introduced late in the course of disease is obviously of clinical relevance. To address this question we transferred T reg lymphocytes into sOVA Tg Rag^{-/-} recipients 6 d after the transfer of naive DO11 cells. Pathogenic effector cells have accumulated in the mice by this time. This late administration of T reg lymphocytes did not prevent the systemic disease (unpublished data).

These studies have explored the roles of cytokines and T reg lymphocytes in a mouse model of a systemic inflammatory disease. This model resembles systemic GvHD, the common feature of both being antigen recognition by transferred T cells in the context of lymphopenia (24). The strengths of the experimental system we have used are that the pathologic immune response is quantifiable because it is caused by T cells of a single specificity reacting to their target antigen, the disease that develops has both systemic abnormalities and local tissue inflammation, and the disease is controlled by endogenously generated T reg lymphocytes. We show that the cytokine IL-17 is the mediator of tissue inflammation in this disease (Figs. 1 and 2). Paradoxically, skin inflammation becomes worse in the absence of Th1 cytokines, presumably because of a concomitant increase in IL-17 production. Worsening of disease in the presence of defective Th1 response has also been noted previously in polyclonal models of GvHD (25, 26). The cross-regulation of Th1 responses and IL-17 has been demonstrated in vitro (19, 20), and our results suggest that this is also true in vivo, but the lineage relationships

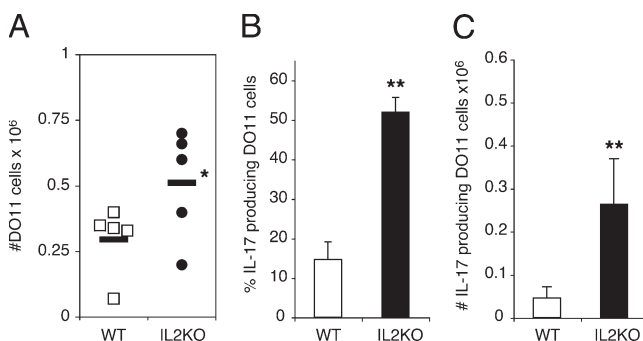


Figure 3. Development of skin-infiltrating IL-17-producing cells in the absence of IL-2. CD4-purified DO11 Rag^{-/-} WT cells were transferred into sOVA Tg Rag^{-/-} WT mice (WT), or DO11 Rag^{-/-} IL-2^{-/-} cells were transferred into sOVA Tg Rag^{-/-} IL-2^{-/-} mice (IL2KO). Ear skin was harvested on day 26 after transfer, and cells were isolated as described in Materials and methods. (A) The total number of CD4⁺KJ1-26⁺ cells recovered from ear skin and (B) the percentage of cytokine-producing KJ1-26⁺CD4⁺IL-17⁺ cells was determined by intracellular staining. Data are pooled from two independent experiments with two to three mice per group. (C) The total number of IL-17-producing KJ1-26⁺ CD4⁺ cells was calculated (*, $P < 0.05$; **, $P < 0.01$).

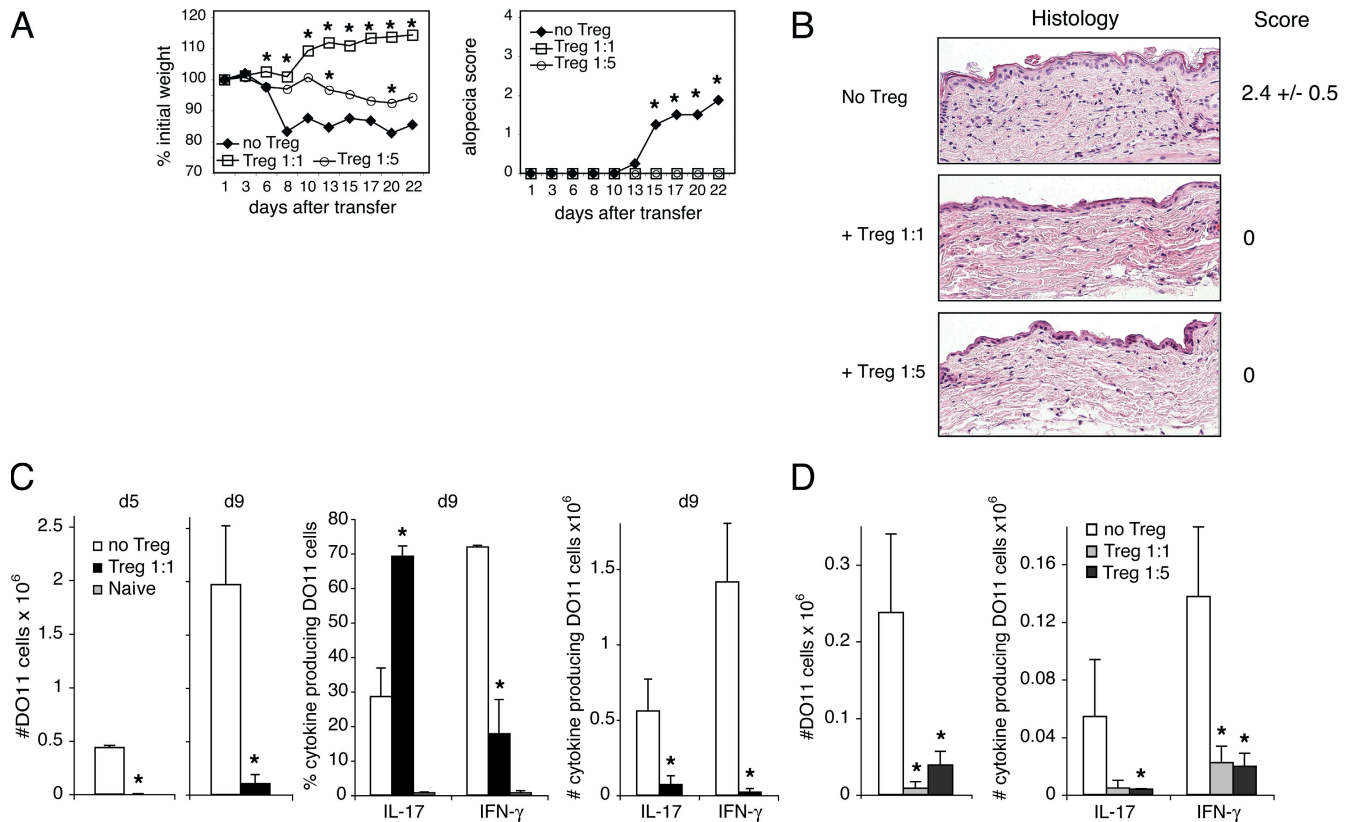


Figure 4. T reg lymphocytes protect from systemic inflammatory disease but do not suppress IL-17 production. CD4⁺KJ1-26⁺CD25⁻ cells were purified from lymph nodes and spleens of DO11 WT Thy1.1⁺ mice, and 10⁶ cells were transferred into sOVA Tg Rag^{-/-} mice on day 0. CD4⁺KJ1-26⁺CD25⁺ cells were isolated from lymph nodes and spleen of DO11×RIP-mOVA Tg mice (Thy1.1⁻), and 10⁶ cells were co-transferred in a separate injection on the same day. (A) Body weight is plotted over time as the percentage of the starting weight before T cell transfer. Data are pooled from four to six mice per group. Alopecia was scored as described in Materials and methods (*, $P < 0.05$ compared with recipients that did not receive T reg lymphocytes). (B) H&E stain of skin sections is shown on day 23 after transfer from one representative mouse in each group. Histological scoring was done as described in Materials and methods, and depicted numbers represent averages of three to four mice per group ± SD. (C) Cells were harvested from

peripheral lymph nodes on day 5 or 9. The total number of isolated CD4⁺KJ1-26⁺ cells on day 5 and 9 (left), the percentage of cytokine-producing effector cells by intracellular staining on day 9 (middle), and total cell numbers of cytokine-producing effector cells on day 9 (right) are shown. All plots are gated on CD4⁺KJ1-26⁺Thy1.1⁺ (*, $P < 0.05$ compared with recipients that did not receive T reg lymphocytes). (D) 0.3×10^6 CD4⁺KJ1-26⁺CD25⁻ cells from DO11 WT Thy1.1⁺ mice were transferred into sOVA Tg Rag^{-/-} mice on day 0, either alone or with CD4⁺KJ1-26⁺CD25⁺ from DO11×RIP-mOVA Tg mice (Thy1.1⁻) at a ratio of 1:1 or 1:5 (T reg lymphocytes to effector cells). Cells were isolated from peripheral lymph nodes on day 23. The total number of isolated CD4⁺KJ1-26⁺ cells (left) and the total cell numbers of cytokine-producing effector cells (right) are shown. All plots are gated on CD4⁺KJ1-26⁺Thy1.1⁺ (*, $P < 0.05$ compared with recipients that did not receive T reg lymphocytes).

between Th1 cells and IL-17-producing cells remain to be defined.

Massive accumulation of IL-17-producing cells in peripheral tissues is independent of IL-2 and also occurs in the absence of T reg lymphocytes. In fact, tissue-infiltrating IL-17-producing cells are increased in the absence of IL-2, and thus in the absence of T reg lymphocytes. This is emphasized by the observation that early after transfer of DO11 Rag^{-/-} cells, which do not contain T reg lymphocytes and at a time point in the disease course when no T reg lymphocytes have developed yet (16), strong differentiation into Th17 has occurred. Our results demonstrate conclusively that the development of Th17 cells is, in fact, increased in the absence of T reg lymphocytes.

Our studies also show that T reg lymphocytes are able to completely prevent the inflammatory disease (13–15, 27). The potent disease-preventing effect of T reg lymphocytes is likely because of the ability of these cells to block initial activation and, as a consequence, antigen-induced T cell expansion after recognition of the systemic antigen. The inability of T reg lymphocytes to ameliorate the disease when given late in the course is consistent with the finding that the principal effect of these cells is to inhibit the initial T cell proliferation and generation of pathogenic effector cells. This result raises the possibility that T reg lymphocytes therapy may be of limited benefit in established immunological diseases. However, it is possible that the propagation of such diseases is dependent on continuous recruitment of new T cells and

their differentiation into pathogenic effectors. If this is indeed the case, T reg lymphocytes may reduce disease severity by inhibiting the continued development of effector cells from newly arising autoreactive precursors. It is also possible that T reg lymphocytes may have little effect on established inflammatory lesions that are caused by local IL-17 secretion, because T reg lymphocytes do not inhibit, and appear to enhance, production of this inflammatory cytokine. Thus, protective or pathologic effects of inflammatory cytokines and the therapeutic effects of T reg lymphocytes vary depending on the location, timing, and context.

MATERIALS AND METHODS

Mice. All experimental mice were used at 5–12 wk of age. Mice in each experiment were age and sex matched ± 2 wk. BALB/c mice were purchased from Charles River Laboratory. Transgenic mice expressing the DO11.10 TCR (DO11), specific for the chicken OVA peptide (OVA_{323–339}) in the context of the MHC class II molecule I-A^d, were obtained from Dr. K. Murphy (Washington University, St. Louis, MO). Some experiments were performed using DO11 cells that had been crossed onto a Rag2^{-/-} background. For some experiments DO11 Rag2^{-/-} mice were bred onto an IL-2^{-/-} background. DO11 \times RIP-mOVA Tg mice have been described (23). DO11 T-bet^{-/-} mice were a gift from Dr. L. Glimcher (Harvard Medical School, Boston, MA), and IFN- γ ^{-/-} mice on a BALB/c background were obtained from The Jackson Laboratories and crossed with the DO11 TCR Tg mice. Soluble OVA transgenic mice (sOVA Tg) on a BALB/c background, expressing a soluble form of OVA in the serum have been described (28). sOVA Tg mice were bred onto a Rag2^{-/-} background. DO11 Thy1.1 mice were obtained from Dr. R. Locksley (University of California, San Francisco, San Francisco, CA).

Scoring for alopecia was done as follows: <1 cm² = 1 point, <2 cm² = 2 points, >2 cm² = 3 points, 50% of body surface = 4 points, complete alopecia = 5 points.

All mice were bred and maintained in our pathogen-free facility in accordance with the guidelines of the Laboratory Animal Resource Center of the University of California, San Francisco. All experiments were conducted with the approval of the Committee on Animal Research of the University of California, San Francisco.

Antibodies and flow cytometry. Lymph node cells, splenocytes, and skin were stained with the clonotypic antibody KJ1-26 (Caltag Laboratories, Burlingame, CA), anti-CD4 (GK1.5, H129.19, and RM4-5), anti-CD25 (PC61, 7AD), anti-CD62L (MEL-14), anti-Thy1.1 (OX-7), anti-Thy1.2 (30-H12), anti-CD3 (145-2C11), anti-GR-1 (RB6-8C5), anti-CD11b (M1/70), and anti-FoxP3 (FJK-16s; eBioscience). All antibodies were obtained from BD Biosciences unless otherwise stated. Antibodies were used as FITC, PE, PE-Cy7, PE-Cy5.5, allophycocyanin, or peridinin chlorophyll protein conjugates. Fc-block (anti-CD16/CD32) was added before staining. Flow cytometric analyses were done on a FACSCalibur with Cellquest Software (both from Becton Dickinson). Percentages were rounded after the first decimal place. Cells were sorted with a MoFlo cell sorter (Cytomation). Propidium iodide (Sigma-Aldrich) was used to exclude dead cells in flow cytometry of skin samples.

For intracellular cytokine staining, DO11 T cells recovered from peripheral lymph nodes, spleen, or skin of transfer recipients were restimulated on mitomycin C-treated BALB/c splenocytes for 14 h in the presence of 1 μ g/ml OVA peptide. Brefeldin A (Epicentre) was added (10 μ g/ml) for the last 2 h of stimulation. Cells were stained for intracellular cytokines IL-17, IFN- γ , IL-4 and analyzed by flow cytometry gating on CD4⁺KJ1-26⁺ cells.

Anti-IL-17 antibody (M210) was provided by Amgen or obtained from R&D Systems (clone 50104). A total of 250 μ g (R&D Systems) or 500 μ g (Amgen) were injected i.p. every 3 d starting on day 4 after transfer. Control Rat IgG was obtained from Jackson ImmunoResearch Laboratory.

Statistical analysis was done using two-tailed *t* test assuming equal variance between the compared groups (**, *P* < 0.01; *, *P* < 0.05).

Cell preparations, purifications, and adoptive transfer. For isolation of T cells from ear skin, the ear was cut off and split into ventral and dorsal half with forceps. The ear halves were minced with a razor blade and incubated at 37°C for 45 min in digestion cocktail containing 0.3% Trypsin from bovine pancreas, collagenase XI (4,100 U/ml), hyaluronidase (260 U/ml), DNase (0.1 mg/ml) diluted in RPMI, containing 10 mM Hepes and 5% FCS (all from Sigma-Aldrich). Digested ears were minced, and isolated cells were washed twice with ice-cold medium containing 10 mM EDTA and filtered through a 40- μ m filter. Further processing was performed as for cells isolated from lymphoid organs.

Histology. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. 5- μ m sections were cut and stained with H&E. Sections were scored blinded using a histological score for dermal inflammation (grade 1 = less than 15% necrotic cells, infiltration less than 20% of dermis; grade 2 = 15–20% necrotic cells, infiltration 20–33% of dermis; grade 3 = 25–50% necrotic cells, infiltration 33–50% of dermis; grade 4 = necrosis in greater than 50% of dermis, infiltration of more than 50% of dermis) and epidermal changes (grade 1 = less than 25% infiltrating cells, 1–2 cell thickening; grade 2 = 25–50% infiltrating cells, 3–4 cell thickening; grade 3 = 50–75% infiltrating cells, 4–6 cell thickening; grade 4 = 75–100% infiltrating cells, more than 6 cell thickening) (29).

Online supplemental material. Fig. S1 demonstrates that peripheral FoxP3-expressing T reg lymphocytes develop from naive CD25⁺ WT, IFN- γ ^{-/-}, and T-bet^{-/-} DO11 cells when transferred into sOVA Tg Rag^{-/-}. Fig. S2 shows that the dermatitis of sOVA Tg Rag^{-/-} recipients is characterized by accumulation of transferred DO11 T cells and GR-1⁺CD11b⁺ granulocytes in the skin. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061341/DC1>.

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